INVESTIGATION ON THE INTERACTION OF DIALDEHYDES AND POLYALDEHYDES ON COLLAGEN*

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ABSTRACT

The interaction of collagen with periodate-oxidized alginic acid was studied in comparison with a number of monoaldehydes (such as formaldehyde and acrolein) and dialdehydes (such as glutaraldehyde, glyoxal, pyruvaldehyde and also dialdehyde starch). Alginic acid was resistant to periodate oxidation, and only about 20 percent of the uronic acid residues of alginic acid were oxidized to the dialdehyde units under conditions in which all the glucose residues of starch were oxidized. A comparative study on the interaction of dialdehydes, polyaldehydes, and monoaldehydes with collagen indicated that dialdehyde- and polyaldehyde-tanned collagen contained a significant amount of free aldehyde groups. The dialdehydes and polyaldehydes (except glutaraldehyde) modified the arginine residues irreversibly. Glutaraldehyde alone behaved differently from other dialdehydes. It modified the lysine amino groups, rather than the guanidino groups, irreversibly.

INTRODUCTION

The reaction of aldehydes with proteins has been of interest for many years, owing to its industrial applications, including tanning and plastics manufacture. Even though extensive work has been carried out on the interaction of collagen with formaldehyde (1), information concerning the reaction mechanism of dialdehydes and polyaldehydes with collagen is rather scanty. In recent years the commercial availability of certain dialdehydes and polyaldehydes has created considerable interest regarding their use as tanning agents.

The tanning properties of glutaraldehyde (2) and dialdehyde starch (3) have been investigated by different workers. The tanning property of periodate-oxidized alginic acid was also reported in an earlier communication (4) from this laboratory. The reaction of certain straight-chain aliphatic dialdehydes with collagen has been investigated by Seligsberger and Sadlier (5). Winheim and Doherty (6)

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from indirect evidence have concluded that, in the tannages with dialdehydes, generally only one aldehyde group reacted, leaving the other aldehyde group free. Seligsberger and Sadlier (5), however, found that no dialdehyde-tanned leather reacted positively to Schiff's reagent. Mardashev and Plekhan (7) treated rabbit muscle with glyoxal, and found the formation of color groups and colorless compounds, which contained free aldehyde groups.

This lack of parallelism made it seem desirable to re-examine some of the aspects of the interaction of collagen with certain dialdehydes and polyaldehydes.

Tanzer (8) has recently reported that, when collagen is reduced with sodium borohydride, the product is a more firmly cross-linked collagen polymer. This was attributed to the reduction of cross-links of the Schiff base type formed by aldehydes present in collagen. It was therefore thought that aldehyde-tanned collagens, in which Schiff base structures are present, may give a more firmly cross-linked collagen polymer when reduced with sodium borohydride.

EXPERIMENTAL

Preparation of Collagen and Modified Collagens.—Purified collagen was prepared from the butt of fresh buffalo hide by the method of Bowes and Kenten (9). About 80 percent of the guanidino groups of collagen were modified by treating with biacetyl (10). All the free amino groups of collagen were destroyed by deamination with nitrous acid by the modified method of Bowes and Kenten (11). About 70 percent of the free amino groups of collagen were converted to guanidino groups by guanidination under the conditions followed by Joseph and Bose (12). In the deguanidinated collagen, where the guanidino groups were blocked by biacetyl, analysis showed that about 17 percent of the ϵ-amino groups were also modified.

Aldehydes.—Formaldehyde (37 percent), glutaraldehyde (25 percent), glyoxal (50 percent), pyruvaldehyde, and acrolein were all reagent grade, and were used without further purification. Dialdehyde starch (75 percent oxidized) was a gift from Miles Chemical Company, U.S.A., and dialdehyde alginic acid was prepared as reported previously (4). The extent of oxidation of alginic acid was determined by two independent methods (namely, the mercurimetric oxidation method (13) and a colorimetric method (14)), and the extent of oxidation was found to be only 20 percent.

Tanning.—All tanning experiments were carried out in carbonate-bicarbonate buffer of pH 9. The collagen powder was soaked overnight before adding the aldehydes. The concentration of aldehyde used was 0.5 M in all cases except dialdehyde starch and dialdehyde alginic acid; in these cases a five percent solution was used. Five-gram portions of collagen were tanned with 100 ml. of the aldehyde solution for 48 hours, at the end of which time they were thoroughly washed and air dried.

Determination of Free Aldehyde Groups in Tanned Collagen.—The carbonyl contents of the different aldehyde-tanned collagen samples were determined by the mercurimetric oxidation method of Ruch and Johnson (13). A 0.5 g. sample of aldehyde-tanned collagen was placed in a conical flask, and 50 ml. of the alkaline potassium mercuric iodide reagent prepared according to Ruch and Johnson (13) was added with constant swirling. The flask was allowed to stand at room temperature (30°C.) for 30 minutes. A 0.1 percent agar solution (50 ml.) was added, and the flask was shaken vigorously for approximately one minute to disperse the mercury precipitate. Then 25 ml. of glacial acetic acid was added with constant agitation during the addition. Approximately 0.1 N iodine solution (50 ml.) was pipetted into the flask, which was shaken vigorously until all the grey mercury precipitate went into solution. The amount of iodine consumed was determined by titration with thiosulfate, using starch as the indicator. A blank with the same amount of untanned collagen was also carried out under identical conditions. The free aldehyde groups were calculated from the amount of iodine consumed.

The Reactivity of the Various Aldehydes to Amino and Guanidino Groups.—The degree of irreversible modification of the arginine and lysine residues by the various aldehydes was determined by hydrolyzing a known weight of the aldehyde-tanned collagen with 6 N hydrochloric acid in a sealed tube for 24 hours, and determining the amount of free arginine and lysine. Arginine was estimated by the method of Macpherson (15), and the total lysine and hydroxylysine by the method of Solomons and Irving (16). The percentages of irreversibly modified lysine and arginine residues were calculated from the losses of these two amino acids.

Reaction of Various Aldehydes with Modified Collagens.—The reaction of the various aldehydes with the modified collagen samples was carried out as in the case of unmodified collagen. After the reaction, they were washed, and the shrinkage temperatures were determined.

Reduction of Various Aldehyde-Tanned Collagens with Sodium Borohydride.—Collagens tanned with the various aldehydes were suspended in carbonate-bicarbonate buffer of pH 9.3, and reduced with excess of sodium borohydride under the conditions reported by Tanzer (8). The reduced products were washed and the shrinkage temperatures were determined.

Absorption Spectra of the Products of Reaction of Glutaraldehyde and Glyoxal with Glycine and Guanidine Carbonate.—Glycine and guanidine carbonate were reacted with a slightly theoretical excess of glyoxal and glutaraldehyde (1.25 moles) at pH 9 for 24 hours, after which the absorption spectra of the four products were recorded.

Shrinkage Temperature.—The collagen powder, after tanning, was thoroughly washed with distilled water. Small fibers were teased out, and the shrinkage temperature was determined by reading in a microshrinkage meter the temperature at which the fibers started to shrink. At least four determinations were made with each sample.

RESULTS

The free aldehyde groups, non-recoverable arginine and lysine, and shrinkage temperature of the various treated collagens are given in Table I. The absorption spectra of the four products obtained by the reaction of glycine and guanidine carbonate with glutaraldehyde and glyoxal are recorded in Figures 1 and 2.

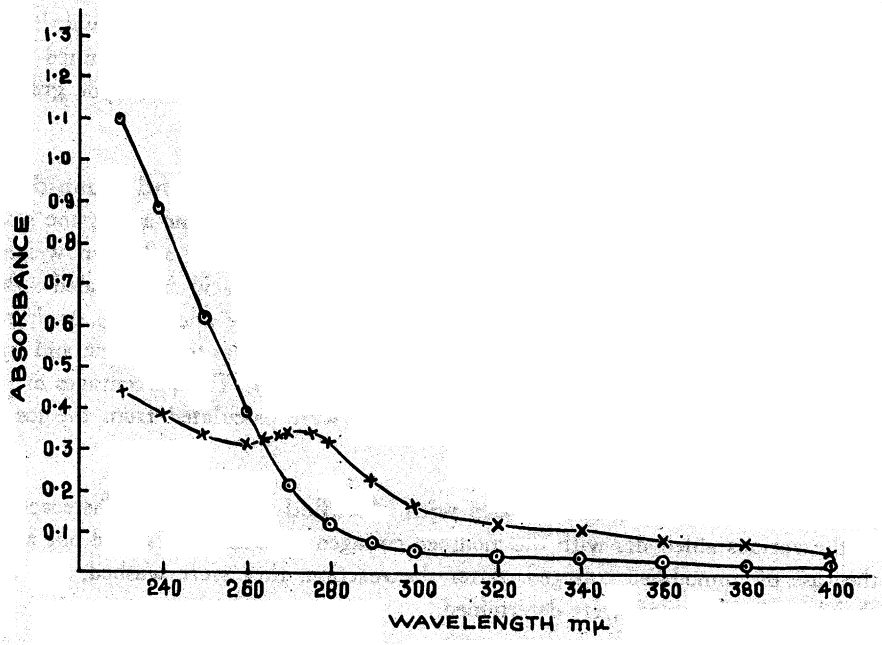

FIGURE 1.—Ultraviolet absorption spectra of the reaction products of glutaraldehyde with glycine and guanidine carbonate.
○ — Glutaraldehyde + guanidine carbonate
× — Glutaraldehyde + glycine

DISCUSSION

From Table I, it can be seen that practically no free aldehyde groups are present in formaldehyde- and acrolein-treated collagens. The very small amount of aldehyde groups detected may be due to the loosely bound aldehydes which are not completely removed on washing. In the case of all other aldehydes, significant amounts of free aldehyde groups are present in the tanned products.

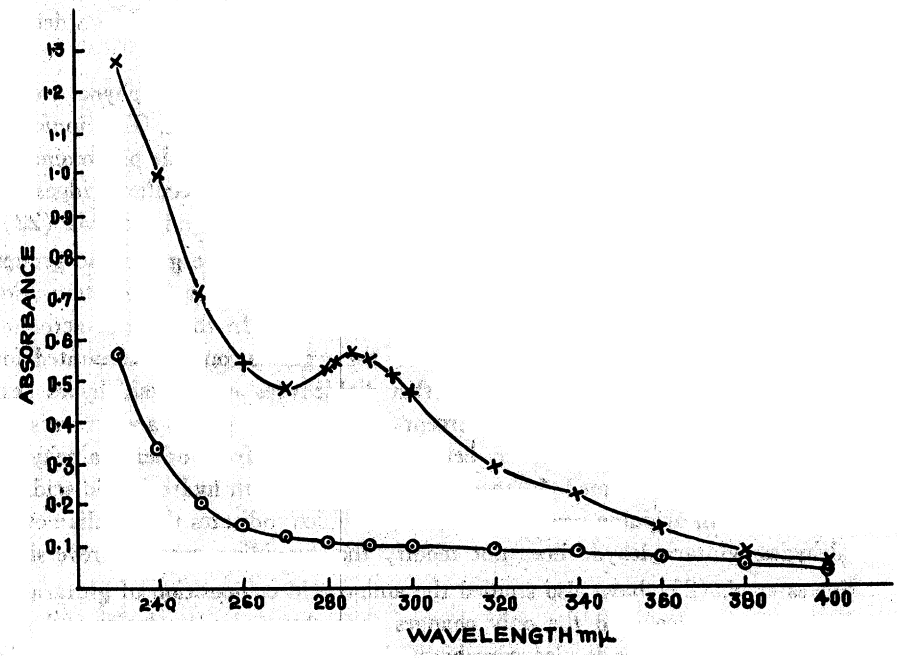

FIGURE 2.—Ultraviolet absorption spectra of the reaction products of glyoxal with glycine and guanidine carbonate.
○ — Glyoxal + glycine
× — Glyoxal + guanidine carbonat

The amount of free aldehyde groups found in dialdehyde starch-tanned collagen in the present study agrees with the value reported previously by Nayudamma *et al.* (3) by the sodium borohydride method of Rankin and Mehltretter (17). The absence of free aldehyde groups in acrolein-tanned collagen indicates that Michael-type additions with the ε-amino groups are not taking place under the tanning conditions employed. It can be assumed, therefore, that the primary reaction of acrolein with collagen takes place through the aldehyde groups, with subsequent completion of cross-linking by reaction at the double bonds.

The presence of free aldehyde groups in the dialdehyde-tanned samples can be explained by the fact that most of these dialdehydes also exist in polymerized forms. In the case of glutaraldehyde, it has been reported recently by Aso and Aito (18) that it may exist in the form of oligomers containing three to four monomeric units. Glyoxal also forms polymeric products under suitable circumstances (19); in water solution it is believed to be a mixture of several hydrated forms. Recent studies have also shown that the polyaldehydes formed from polysaccharides by periodate oxidation exist as cyclic acetals and hydrates in complex equilibria (3, 20, 21). When such polymeric structures containing several aldehydes or potential aldehyde groups interact with collagen, it is quite possible that

steric factors and nonavailability of protein groups might prevent some aldehyde or potential aldehyde groups from taking part in the reaction.

In the case of glutaraldehyde tannage, the amount of free aldehyde groups detected is much less than that found with the other dialdehydes. This indicates that reaction of both aldehyde units on each glutaraldeyde molecule predominates, owing to the formation of either cyclic structures or intramolecular bridges between neighboring sites, as suggested by Thies, Cuthbertson, and Yoshida (22).

From Table I, it can also be seen that the reactivity of the guanidino groups and the nature of the compounds formed by these groups during interaction with the different aldehydes vary from aldehyde to aldehyde. In the case of acrolein- and formaldehyde-tanned collagen, almost all the arginine could be accounted for in the acid hydrolysates. In the case of other dialdehydes and polyaldehydes (except glutaraldehyde), about 60–80 percent of arginine residues are irreversibly modified. Glutaraldehyde, however, behaved differently from other dialdehydes. When glutaraldehyde-tanned collagen was hydrolyzed with hydrochloric acid, no significant loss in arginine was observed, a fact which indicates that, unlike other dialdehydes, glutaraldehyde does not modify the guanidino groups irreversibly.

Bowes *et al.* (23) have also studied the amino acid composition of glutaraldehyde-tanned collagen, and the only changes they observed were losses of lysine and hydroxylysine. This is also corroborated by the results obtained for the irreversible modification of lysine residues, where 58 percent of the lysine residues are irreversibly modified by glutaraldehyde. In the case of formaldehyde-tanned collagen, there was no loss in lysine and arginine contents. Some of the other dialdehydes, such as pyruvaldehyde, also modified the lysine residues irreversibly. Of the various aldehydes studied, dialdehyde starch showed the maximum ir-

TABLE I

FREE ALDEHYDE GROUPS, NONRECOVERABLE ARGININE AND LYSINE, AND SHRINKAGE TEMPERATURE OF VARIOUS ALDEHYDE-TREATED COLLAGENS

Nature of Treatment	Free Aldehyde Groups (mmoles/gm.)	Non-recoverable Arginine %	Non-recoverable Lysine %	Shrinkage Temperature		
				Deguanidinated	Guanidinated	Deaminized
Formaldehyde	0.072	13	0	67	71	54
Pyruvaldehyde	0.41	70	43	—	60	—
Acrolein	0.00	9	20	62	67	48
Glutaraldehyde	0.2	15	58	63	49	53
Glyoxal	0.44	61	17	61	69	56
Dialdehyde Starch	0.5	87	30	61	75	53
Dialdehyde Alginic Acid	0.36	35	3	59	—	50
None	—	—	—	53	47	53

reversible modification of arginine residues. It is, however, not very clear why glutaraldehyde behaves differently from other dialdehydes in its reactivity to arginine residues. Probably in this case the two amino groups of arginine modified with bifunctional glutaraldehyde may be less stable than the single amino group of modified lysine.

Filachione *et al.* (24, 25) have reported the presence of some unknown substances having a strong absorption maximum at 265 mμ in the hydrolysates of glutaraldehyde-tanned collagen. In the present study, also, it has been found that, when glutaraldehyde is reacted with glycine at pH 9, the reaction product gives an absorption maximum at about 270 mμ, as shown in Figure 1. When glutaraldehyde was, however, reacted with guanidine carbonate under identical conditions, the product did not show any absorption maximum in the ultraviolet region (Figure 1). On the other hand, the reaction product of guanidine carbonate and glyoxal showed an absorption maximum at 286 mμ under identical conditions. It appears, therefore, that the guanidine carbonate is not reacting appreciably with glutaraldehyde at this pH value. It is also interesting to note that guanidinated collagen, in which most of the amino groups were converted to guanidino groups, did not react with glutaraldehyde to form cross-links, whereas all the other aldehydes showed the ability to form cross-links with guanidinated collagen. Guanidinated collagen also failed to show any increase in shrinkage temperature when reacted with glutaraldehyde, even at a high alkaline pH (pH 10.5). These results, therefore, lend further support to the suggestion that, in the tannage with glutaraldehyde, the guanidino groups of collagen are not taking part in the reaction. It is surprising that deaminized collagen, which contains guanidino groups and no amino groups, does not form cross-links with the different aldehydes, whereas guanidinated collagen, in which guanidino groups and only few amino groups are present, is able to form cross-links except in the case of glutaraldehyde. These results show that guanidino groups properly spaced for the formation of cross-links are present only in guanidinated collagen, and not in deaminized collagen. The results obtained with deaminized collagen further suggest that the formation of cross-links in collagen by different aldehydes takes place through the mediation of amino groups. When guanidino groups occupy the positions of these amino groups, the collagen is still able to form cross-links with all aldehydes except glutaraldehyde.

Sodium borohydride (26, 27) has been reported to be capable of reducing Schiff bases. However, when collagen tanned with the various aldehydes was subsequently treated with sodium borohydride, no improvement in the properties of the various aldehyde-tanned collagens was observed. Only in the case of dialdehyde starch-tanned collagen could an increase in shrinkage temperature of about 8°C. be observed on reduction with sodium borohydride. It appears, therefore, that the Schiff base structures are either absent in most of the aldehyde-treated products, or, if they are present, their reduction does not in any way contribute

to an improvement in the physical properties of the leathers. Tanzer (8) has, however, reported that, when native tropocollagen is reduced with sodium borohydride, the product is a firmly cross-linked collagen, as shown by its solubility, subunit composition, and thermal shrinkage. It appears, therefore, that the aldehyde linkages present in native collagen and those formed in aldehyde tannage are different in nature.

The results obtained in the present study show that the various aldehydes do not react with the various functional groups of collagen in an identical fashion. Even though the precise nature of the cross-links formed in the case of the different aldehydes is yet to be established, the results suggest fairly conclusively that the amino groups are involved in cross-link formation in almost all the aldehvde-treated collagens.

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